REVIEW ARTICLE

# Proteomics: methodologies and applications to the study of human diseases

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## Summary

Proteomic approach has allowed large-scale studies of protein expression in different tissues and body fluids in discrete conditions and/or time points. Recent advances of methodologies in this field have opened new opportunities to obtain relevant information on normal and abnormal processes occurring in the human body. In the current report, the main proteomics techniques and their application to human disease study are reviewed.

**Keywords:** Proteomics; neoplasms; polyacrylamide gel electrophoresis; mass spectrometry; diseases.



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## Introduction

In the search for molecular markers that could assist in the early diagnosis and treatment of several human diseases, including cancer, many studies have focused on changes in genes, their transcripts, and protein products involved in important cellular processes.

Recent methodological approaches allowing a wide gene expression analysis include cDNA[1] microarray technique, serial analysis of gene expression (SAGE)[2], and large-scale sequencing techniques using state-of-the-art equipment[3]. The study of gene expression by using these techniques gives a molecular profile and provides opportunities to identify important changes occurring at the RNA level. However, transcript analysis is impaired by a susceptibility to breakdown and a nonconformity between the transcript and the protein concentration[4]. Moreover, information on processes modulating protein function and activity, such as post-translational changes, protein-protein interactions, transportation, and breakdown are lost in RNA analysis[5]. Thus, it is important that, in parallel to data derived from the genome and clinical data, information on protein differences across normal and altered tissue and/or body fluids are also collected so that the mechanisms involved in human disease are understood, with consequent benefit for patients.

To identify and understand the differences, it is crucial to know the set of proteins encoded by the genome and defined as the proteome[6]. Indeed, the proteome is not only the sum of products translated from genomic sequences, but it also includes proteins resulting from post-transcriptional and post-translational processing, as well as complexes formed by these biomolecules[7]. In addition to its great complexity, the proteome is dynamic and its profile changes according to physiological status and phases of cell differentiation. Some estimates suggest that over a million different types of proteins are present in cells, tissues, and body fluids in discrete conditions and/or time points[8]. The term proteomics regards the study of this set of molecules, that are directly or indirectly responsible for controlling all or nearly all biological processes. As well-defined by Valledor and Jorrin[9], proteomics is the descriptive and quantitative study of proteins, from those in a subcellular organelle to those in an ecosystem, as well as their variations in the population, changes in response to the environment or resulting from normal or altered development, and modifications and interactions with other proteins.

## Methodology in proteomics

Many of the techniques employed in proteomics focus on the identification of biomarkers, but they are limited for direct medical applications. Other techniques have a potential for automation and use in clinical routine with diagnostic purpose, and allow the analysis of many kinds of samples and changes in the pattern of protein expression associated with a disease. Overall, methodologies employed in proteomics (Figure 1) may be classified into bottom-up and top-down types. The former, also termed shotgun[7], includes liquid chromatographic separation of peptides derived from tryptic digestion of complex protein solutions, followed by mass spectrometry (MS) analysis. The top-down method, in contrast, is a process in which intact proteins (not peptides) undergo MS analysis. Bottom-up approaches have many advantages, such as sensitivity and reproducibility, even for complex proteomes, such as cell lysates and serum. However, the responses obtained are fragments of a whole, and although protein identification based on a few peptides is possible, post-translational modifications are not recognized. In addition, a peptide may either be lost during chromatography, or appropriate mass spectra may not be generated. Thus, top-down proteomics has received a great deal of attention from the scientific community[10].

Combining these approaches with other processes, such as subcellular fractionation or protein immunoprecipitation, can be quite effective to enrich a sample with low-abundance compounds or cell organelles of interest[11]. Fresh samples constitute the first choice in these studies, but as they are difficult to obtain, particularly in rare diseases, some methods have been developed for paraffin embedded specimens[12].

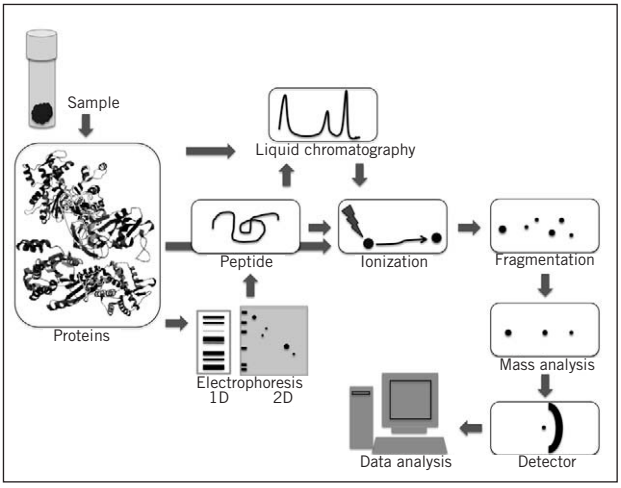


**Figure 1 –** Different methodologies can be combined in proteomic studies. Methodologies more commonly used involve protein extraction from the sample, separation by one- (1-D) or two-dimensional (2-D) electrophoresis and/or liquid chromatography, ionization, fragmentation, peptide analysis and detection, and data analysis.

### Protein separation by one- and two-dimensional electrophoresis

To separate proteins by one- (1-D) and two-dimensional (2-D) electrophoresis, the molecules must be initially isolated from biological materials, such as tissues and body

fluids. Appropriate protein extraction is crucial to obtain good electrophoretic results. As a function of the various types and sources of biological samples, the extraction procedure needs individual optimization. In most cases, proteins need to be solubilized, disaggregated, denatured, and treated with disulfide bond reducing agents[13].

In typical 2-D electrophoresis, proteins are separated in two consecutive steps. At the first step, termed isoelectric focusing (IEF), molecules migrate in a polyacrylamide gel with an immobilized[14] or amphoteric buffer-generated[15] pH gradient until they reach a point (pH) in which their charge equals zero (isoelectric point – IP). At the second step, proteins undergo an electrophoresis whose direction is perpendicular to IEF in polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE), being separated according to their molecular mass. This second step is similar to a 1-D electrophoresis, in which molecules are directly applied to SDS-PAGE and separated according to their size.

In order to make protein bands or spots visible (1-D and 2-D, respectively), gels are stained by Coomassie blue, silver nitrate, or other commercially available dyes. In 2-D gels, 100 to 2,000 spots can be visualized, each one of them containing one to several proteins, and some post-translational changes are easily detected as vertically or horizontally aligned trains of spots. After gel image digitalization and use of computer tools, the background material is extracted, the spots are compared, and the data are normalized and statistically analyzed for protein volume or intensity quantification[16]. A simpler protocol is used for 1-D gels, whose bands of interest or full runs are sliced and analyzed[17]. Proteins found in these slices or in 2-D gel spots are digested into peptides by trypsin, with cleavage being made after arginine or lysine residues.

Many modifications have already been made in the 2-D original protocol. One of the most recent and popular is based on labelling cyanine fluorescent dyes reacting with lysine or cysteine residues. This labeling gave rise to a technique, the Fluorescent 2-D Differential In-Gel Electrophoresis (2-D DIGE)[18], allowing for the analysis of two protein samples marked with different fluorochromes in the same gel, thus reducing inter-gel variation and improving the efficiency and accuracy of the method.

Although 1-D and 2-D electrophoresis techniques can generate much information, they have limitations. One of the most important limitations is the presence of some proteins in elevated concentrations, especially in certain body fluids, which makes electrophoretic migration of less abundant proteins more difficult. Another limitation is that the extraction of intact proteins from the gel for top-down analysis is difficult, but some attempts to circumvent this problem have been made[10].

### Peptide fractionation by liquid chromatography and identification by mass spectrometry

Several types of chromatography are used to reduce the sample complexity or to complement protein and peptide separation by electrophoresis. In liquid chromatography (LC), the analyte is dissolved in a liquid phase without chemically interacting with it, and percolates a stationary phase usually packed into one[19] or several columns with different stationary phases, such as in Multi-dimensional Protein Identification Technology (MudPIT)[20,21].

Although the analytes are characterized by molecular mass (and IP in 2-D) and purified or fractionated by chromatography, they need to be identified; this is performed by mass spectrometry[22]. The technique basically consists of ionizing a compound and evaluating the ion mass/charge (m/z) ratio. The equipment comprises a ionization source, one or two mass analyzers and a detector. The first component is used to generate peptide or protein ions, usually transferring protons ($H^+$) to the molecules without modifying their chemical structure. The ion is accelerated by an electric field and separated by m/z in a mass analyzer, or it is selected according to a previously determined m/z, being fragmented in a tandem process (MS2 or MS/MS). Finally, the ions pass through the detector, which is connected to a computer with data analysis software[19].

### Ionization methods

Currently, two main ionization methods are available and used in proteomics, Matrix-Assisted Laser Desorption/Ionization (MALDI) and Electrospray Ionization (ESI), with the former being employed for solid state samples and the latter for liquid state samples (Figure 2). In MALDI, peptides are co-crystallized with an organic matrix, usually alpha-cyano-4-hydroxycinnamic acid. After laser bombardment, the matrix sublimates and its ions transfer the charge to analytes, resulting in peptide ion formation[23]. One MALDI variant termed Surface-Enhanced Laser Desorption/Ionization (SELDI) is usually employed to analyze a low-molecular-weight proteome and uses several matrices or chips that explore the chromatographic and biophysical characteristics of different proteins. These chips can exhibit hydrophobic surfaces; ion exchange surfaces or surfaces with immobilized metallic ions; or even antibodies, receptors, enzymes, and ligands with high affinity for specific proteins[24]. Thus, after washing out unbound compounds, a matrix is added to the chip surface and spectra are acquired through laser ionization. Another MALDI variant is Imaging Mass Spectrometry (IMS), allowing peptide and protein mass data to be obtained directly from biological tissue sections. This method offers important advantages over immunohystochemical analysis, including speed and independence from antibody use[25].

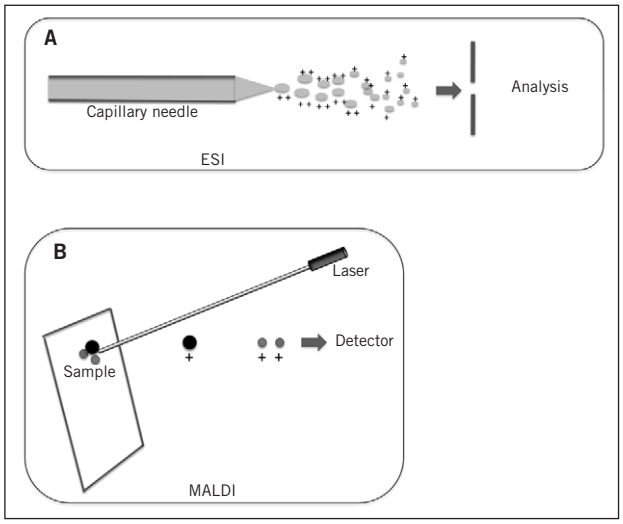


**Figure 2 –** Ionization methods. (**A**) ESI method: an aqueous solution containing the analyte is forced through a capillary needle and ejected as a spray with highly charged droplets, which generate analyte ionized forms after solvent evaporation. (**B**) MALDI method: peptides are co-crystallized in an organic matrix and ionized after laser bombardment.

In contrast with MALDI, in ESI an aqueous solution with the analyte is forced to pass through a capillary needle undergoing high voltage. The solution is ejected as a spray with highly charged droplets that generate analyte ionized forms after the solvent is evaporated by a heated inert gas flow[26].

### Types of analyzers

Regardless of the ionization method, the ion molecular mass is assessed in an analyzer after passing through a vacuum chamber. The most common analyzers are Time Of Flight (TOF), quadrupole (Q), and ion trap (IT)[19].

In TOF analyzers, the ions resulting from the first step are accelerated by a potential between two electrodes and pass through a vacuum tube at a speed that is inversely related to their mass. When the ions reach the detector, the time elapsed from the ionization up to the detection is used to derive the m/z value. In fact, the detector converts the signal of the ion passage into an analog signal, which is read and interpreted by a workstation. The final result is a plot of m/z versus intensity (ion count), usually referred to as MS spectrum[27]. The generated signals are compared with information available in databases, such as MASCOT[28] and SEQUEST[29], to identify the protein of interest.

One of the limitations of the MALDI-TOF system is that the detection of low-molecular-weight proteins is difficult, as they generate few peptides. The system is also not able to detect more than one component in a mixture. TOF analyzers may be combined with Q analyzers, which have a set of four-rod electrodes and work as mass filters so that their performance can be improved. Between these electrodes, an electric field ensures that only ions with a certain m/z ratio travel to the detector, while the others are deflected[30].

The IT analyzers filter and entrap ions of interest in a tridimensional electric field and these are gradually released in an m/z ascending order[31]. Fourier Transform Ion Cyclotron Resonances (FT-ICRs) are ion traps with an additional magnetic field forcing ions to exhibit a circular movement with high frequency cycles. The analyzer determines the m/z ratio from the cyclotronic movement frequency by using the Fourier transform[19]. Orbitrap is another type of IT analyzer wherein ions oscillate along and around a single spiral electrode. This oscillation frequency is directly related to the square root of the m/z ratio and can be determined with high accuracy[32,33]. This technology has migrated towards hybrid systems with two independent mass spectrometers that combine, for example, an ion trap and an orbitrap, or an ion trap and a FT-ICR.

### Protein identification

After determining the m/z ratio of the intact peptide, its sequencing can be performed through a second MS event, as described above: more abundant peptides are specifically selected and undergo fragmentation by collision with an inert gas (collision-induced dissociation – CID) or by electron transference (ETD); the latter is advantageous for preserving protein post-translational modifications in top-down analysis. The parental peptide fragmentation occurs predominantly along its skeleton, usually between the carbonyl oxygen and the amide nitrogen, thus generating two ion groups termed y and b. The resulting MS/MS spectrum is, in fact, a list of m/z ratios for distinct fragments whose mass differences correspond to single amino acids. Evaluating these size-ascending fragments from the N-terminus (b ion series) or the C-terminus (y ion series) allows for deducing the peptide sequence. With the results for several peptides, the protein can be identified[33].

## Quantitative methods

In recent years, several methods of absolute and relative protein quantification in samples assessed by MS have been developed. Originally, the only available platform was 2-D gel, a technology that allows for the assessment of hundreds or thousands of protein spots, despite its limitations[9]. More recently, some methods use protein or peptide labeling by isotopes or other reactants identifiable by MS, such as linkers with heavy isotopes in Isotope-coded Affinity Tag (ICAT)[34], Isobaric Tags in Isobaric Tags for Relative and Absolute Quantification (iTRAQ)[35] and in vivo labelling of proteins with amino acids containing nonradioactive isotopes in Stable Isotope Labeling with Amino acids in Cell Culture (SILAC)[36]. In short, two samples to be compared are covalently modified by isotopes (e.g., $^{1}H$

versus $^{2}H$, $^{12}C$ versus $^{13}C$) and differences in protein quantities are determined by the intensity ratio of differentially labeled peptides.

Labeling-free quantification methods have also been developed thanks to technological advances in liquid chromatography and mass spectrometry systems, as well as in bioinformatics tools for data interpretation[37]. As an example, the intensity of peaks of mass spectra generated by peptide ions is correlated with abundant protein. The same is observed regarding the count of MS/MS spectra, as Old et al.[38] noted.

### Applications in the study of human diseases

Although the proteome fraction possibly identifiable by using the approaches described above has been growing, the analysis is still incomplete even in simpler cells, especially for low-abundance (such as receptors, signal transducers, and regulators), basic, and hydrophobic proteins, as well as membrane proteins or those with molecular mass above 150 kDa or below 10 kDa[39]. This picture is supposed to change because methodologies and technologies in this field have had great advances over the last years and have reached high levels of resolution and application potential. As expressed by Walsh et al.[40], proteomics has moved from the question "what?" towards questions involving "when, where, how, and how much".

However, what are the benefits of proteomic studies for human disease management? The literature on this subject is extensive, and many relevant data have already been obtained, including the characterization, albeit partial, of proteins in different tissues and conditions, and of subproteomes, such as phosphoproteomes[41] and glycoproteomes[42].

However, specific and sensitive biomarkers are not easily identified through proteomic approaches. This is revealed, for example, by data obtained from head and neck, breast, colon, and ovarian cancers[43-45]; although they are different conditions, they show similar changes. Only one screening test (OVA1) developed with the SELDI-TOF methodology for ovarian cancer has been approved[46,47].

Tissues affected by most human diseases are not easily accessible for analysis and they will be unlikely to be used in routine analysis. One of the main limitations is cell heterogeneity, possibly leading to inaccurate results if a thorough histopathological study is not performed. Laser microdissection overcomes this problem, but it generates a reduced number of cells and introduces extra sample handling. In contrast, body fluids have characteristics that surpass these limitations and are appropriate for developing low- or less-invasive diagnostic and prognostic tools. Moreover, they are especially appropriate when longitudinal monitoring is required[32]. Prostate specific antigen (PSA) in prostate cancer, and tyrosine kinase receptor CD340 in breast cancer are good examples that proteins released into the blood by diseased tissues can be illness indicators when the concentration is altered[48]. However, there are many technical challenges in the use of these biological materials, with the most important being the complexity, the dynamic characteristic of the protein composition, and the need to analyze a great number of patients to determine intra- and inter-individual variability for a potential marker. In addition, one marker alone would hardly have enough sensitivity and specificity for prediction or diagnosis for developing clinical tests; protein panels associated with specific conditions will likely be required.

Several organ-specific body fluids have already been characterized aiming at clinical use, such as urine for Anderson-Fabry disease[49]; cerebrospinal fluid for multiple sclerosis[50] and amyotrophic lateral sclerosis[51], Alzheimer disease[52], Creutzfeldt-Jakob disease[53], and Parkinson disease[54]; bronchioalveolar lavage for chronic obstructive pulmonary disease[55]; synovial fluid for osteoarthritis[56]; tears for keratoconus[57]; and nipple aspirate for breast cancer[58].

## Body fluids: saliva

Saliva is a biological material well studied by proteomic approaches. Comprising a mixture of components secreted by salivary glands and derived from the blood, it is likely the most accessible fluid in our body[59]. It plays an important role in supporting oral health by participating in processes such as dental enamel remineralization, defense against microorganisms, lubrication, digestion, and pH and taste modulation[59-62]. These attributes result from the component characteristics, including proteins, hormones, small molecules (such as urea), and electrolytes (such as calcium, bicarbonate, phosphate, and fluoride)[59]. Salivary proteins have been studied by traditional and proteomic biochemical techniques, and hundreds have already been identified both in total saliva and in individual gland secretion, although those expressed in low levels certainly have not yet been detected[57,63-89].

The great interest in saliva as a fluid for diagnosis has led to a standardization of collection and storage processes[90] mainly because several factors affect saliva flow and composition. Among these factors, physiological status, drugs, foods, odors, circadian rhythm, gender, age, blood composition, and degree of salivary gland activity[91,92] are noticed. Thus, flow parameters and salivary composition have been explored in monitoring hormone[93] and drug[94] levels, exposition to environmental pollutants[95] and infectious agents[96], and disease monitoring, including periodontitis[97], diabetes mellitus[98], cystic fibrosis[99], Sjögren syndrome[100], salivary gland diseases[101], and breast[102,103], ovarian[104], and oral[105,106] cancer. Regarding oral cancer, the anatomic site offers saliva an important advantage over other fluids, in addition to the noninvasive characteristic and the compatibility with proteomic approaches. Being

in contact with the affected tissue, thus receiving proteins secreted or derived from dead cells, its potential use ranges from early detection[107] to aggressiveness prediction and prognostics[108].

Although many studies have identified salivary biomarkers in local and systemic diseases, their validation in large sample groups is not available, and data from different authors on the same disease show conflicting results[109]. Nevertheless, a number of interesting associations have been reported. For example, elevated transferrin levels were observed in patients with oral carcinoma, correlated with tumor size and stage. Assays for ELISA were highly specific and sensitive for early detection of this carcinoma, which makes transferrin a promising marker[110]. This protein is essential for cells with a high proliferation level, and is involved in DNA synthesis and transduction paths of mitogenic signals[111].

Regarding the potential of therapy response prediction, recently Vidotto et al.[112] observed that the levels of some salivary proteins in patients with head and neck carcinoma revert to a pattern similar to that observed in healthy individuals after treatment. Among these proteins, two of them (PLUNC and ZN-alpha-2-GP) are related to inflammation, which is frequently found in these tumors.

## Body fluids: serum/plasma

Although saliva and other body fluids allow for obtaining relevant data for proteomic analysis, mainly in diseases affecting specific tissues and organs, no question remains that serum and plasma are much more comprehensive. These blood fractions are among the most important sources of biological markers and can provide rich information about physiological and pathological processes[113]. Their analysis for diagnostic purpose is well known, and both fractions are similar in composition. However, plasma appears to be more stable and more appropriate than serum to assess low-molecular-weight proteins. On the other hand, serum is the material of choice for several tests because plasma anticoagulants interfere with some methods employed[44].

Only 22 proteins, such as albumin, transferrin, haptoglobin, immunoglobulins, and lipoproteins make over 95% of serum/plasma proteome. Many cell proteins, in contrast, enter circulation in very reduced levels[114]. As an example, albumin is found in blood in a millimolar ($10^{-3}$ mol) concentration, while other proteins, such as cytokines, have an activity in concentrations between $10^{-12}$ mol and $10^{-9}$ mol[115]. This smaller group certainly includes disease biomarkers[116] whose detection unfortunately may be interfered with by very abundant proteins. In a review by Kawashima et al.[117], various depletion methods are used; however, they often result in the removal of low-molecular-weight proteins (Figure 3).

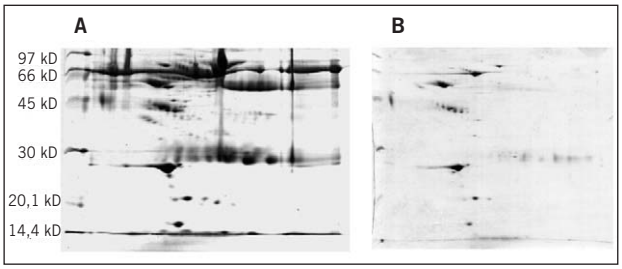


**Figure 3 –** Gels resulting from 2-D serum protein electrophoresis from patients with neurofibromatosis. (**A**) Without and (**B**) with depletion of more abundant proteins.

Schiess et al.[48], by comparing known markers with proteins identified by proteomic approaches, observed plasmatic concentrations with very different orders of magnitude. While levels of markers such as PSA and CD340 are in the range of pg to ng/mL, the levels of classic plasma proteins are in the order of μg to mg/mL. These data show the need for advances in technology so that detection limits reach lower concentration levels[114]. Recently, measurements by Selected Reaction Monitoring (SRM) in mass spectrometry have been used to overcome these difficulties, as they focus on *a priori* selected protein sets which have generated very consistent data[118,119], especially when abundant component depletion and fractionation are combined[120].

Despite these limitations, many data have already been obtained from the serum/plasma of patients with diabetes[121,122]; autoimmune diseases[123]; heart[124] and infectious[125] diseases; Parkinson[126] and Alzheimer[52] diseases; endometriosis[127]; bladder[128], head and neck[129-132], colon[133,134], esophagus[135], stomach[136], liver[137,138], breast[139,140], pancreas[141,142], prostate[143,144], lung[145,146], and kidney[147-149] tumors; and also pregnant women with fetuses with Down syndrome[150].

Although the number of publications is high, only one screening test developed from proteomic approaches has been approved (OVA1). The test analyzes a protein panel (CA125, transthyretin or prealbumin, apolipoprotein A1, beta-2-microglobulin, and transferrin) and, when combined with clinical and imaging evaluation, presents a sensitivity higher than 90% for pre-surgical assessment of ovarian cancer risk[46,47].

## Final considerations

Many factors affect the results of proteomic analysis, especially regarding body fluids. Patient and environmental characteristics are among these factors[44,151]. In the pre-analytical stage, material processing introduces other variables, such as collection method, type of storage, and initial sample treatments. Likewise, proteolytic breakdown products generated in the analytical stage influence the results if effective protease inhibitors are not used. Breakdown by catabolism is equally important[152], although low-molecular-weight fragments are not always nonspecific, such as those derived from transthyretin[153] and osteopontin[154].

The clinical aspect of proteomic studies has also faced a few challenges. One of them is prospective analysis of representative and well-characterized populations to acquire statistical power and surpass the limitations resulting from individual variability and biological material processing.

Despite these challenges, there is no doubt that the results of proteomic approaches are potentially useful in several clinical research areas, such as diagnosis, therapy response monitoring, endpoint prediction, disease subtype classification, risk determination, characterization of metabolic pathways, biomarker quantification, and therapeutic target generation[40].

In recent years, many important biological questions have been answered by proteomics and hundreds of candidate biomarkers have been introduced. However, few markers have surpassed the identification stage. Their successful application to clinical practice will depend on sensitive platforms; development of protein panels; and collaborative studies including physicians, epidemiologists, molecular biologists, and bioinformaticians with a relevant clinical issue and well-defined recruitment and characterization parameters for patients and samples.

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